Synthesis and spin-trapping behaviour of glycosylated nitrones

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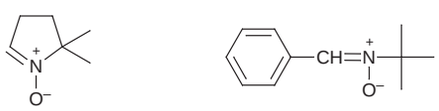
In the course of our investigation dealing with the vectorisation and cell targeting of biocompatible spin traps, the synthesis and spin trapping behaviour of two glycosylated nitrones, LAMPBN (*N*-[4-(lactobionamidomethylene)-benzylidene]-*N*-*tert*-butylamine *N*-oxide) and LAMPPN (diethyl [1-methyl-1-[4-(lactobionamidomethylene)-benzylidene]azinoylethyl]phosphonate), have been studied and their interactions with yeast bearing specific membrane lectins have been successfully assessed.

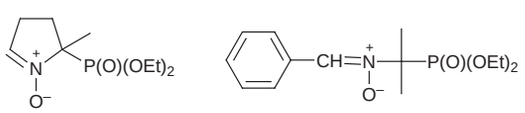
Introduction

Studies on biological radicals and especially oxygen-centred radicals have become of considerable interest in the biomedical area. Involved in host defence, these radicals also appear as important mediators in many clinical disorders and tissue injury.[1] They are implicated in ischemia–reperfusion injury[2a] observed, for example, in myocardial infarction,[1,3] brain ischemia,[1,4] organ transplants,[1,5,2a] glaucoma and cataractogenesis.[1,5,6] They are also suspected to be involved in inflammatory–immune disorders such as emphysema[6] and adult respiratory distress syndrome.[6] Of the methods available for assessing free radical formation in biological systems, electron spin resonance spectroscopy (ESR) associated with spin trapping appears the most appropriate and has been widely used.[7] Nitrones have emerged as the most powerful spin traps for biological applications[2c,8] and out of several nitrones, the cyclic 5,5-dimethylpyrrol-1-ine-*N*-oxide (DMPO) and *N*-(benzylidene)-*tert*-butylamine *N*-oxide (PBN) have received the most attention. However, as has been emphasised by different authors,[9] the use of these spin traps is not without its limitations. The reaction of DMPO with superoxide radical ($O_2^{\bullet -}$) or hydroperoxyl ($HOO^{\bullet}$), which is often assessed as the prime radical of the radical reaction chain, is rather slow, having a second-order constant ranging from 10 mol^{-1} dm^3 s^{-1} at pH 7.8[9a] to 1.2 at pH 7.4.[10] Furthermore the superoxide spin adduct is short-lived[11] and undergoes a rapid chemical conversion in an aqueous environment to the hydroxyl radical spin adduct. DMPO has been applied to intracellular spin trapping[12] but its low partition coefficient might limit this use.[13]

The much more lipophilic PBN has a better subcellular biodistribution than DMPO,[13b,14] but in aqueous solution the hydroxyl spin adduct (PBN–OH) lasts only a few seconds[15] while the superoxide spin adduct decays even more rapidly.[16] PBN is particularly useful for trapping carbon-centred radicals[17] which can result from the attack of cell or membrane molecular components by the oxygenated species.[2d,18] We recently reported that the superoxide spin adduct obtained with 2-diethylphosphono-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide (DEPMPO) is much more persistent than its DMPO analogue.[19] Moreover for DEPMPO adducts, additional information on the trapped radical is obtained from the large phosphorus coupling observed. Encouraged by these results, we prepared *N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide (PPN) and we found that PPN is a better spin trap than PBN for the superoxide radical.[20] Nowadays, it appears that one important limitation to the application of spin trapping to biological systems results from insufficient knowledge concerning the biodistribution, the membrane crossing ability and the biological target of the routinely used spin traps. To produce a suitable pharmacomodulation, one of the possibilities is to graft the spin traps to a natural or synthetic amphiphile biocompatible carrier. This carrier, which could be a molecular,[21] macromolecular[22] or supramolecular[23] delivery system, will modify the hydro- or lipophilicity of the nitrone and thus its biodistribution. Furthermore, it could ensure specific cell targeting *via* its functionalization with ligands such as antibodies, peptides or carbohydrates.[24] It is well known that membrane lectins are glycoproteins which serve as receptor sites for carbohydrates.[25] Galactose, *N*-acetylgalactosamine, mannose and mannose phosphate receptors have been identified on hepatocytes and cells of the reticulo-endothelial system.[26] Thus, it seems to be of interest to use the mechanism of recognition of glycosylated carrier by membrane lectins to increase the graft drug activity. In the course of our project dealing with the use of glycosidic amphiphiles as specialised spin traps, we decided to combine firstly cell targeting and spin trapping properties. We report herein the synthesis of new glycosidic nitrones derived from PBN and PPN, a study of their ability to trap the superoxide radical in an aqueous environment, and preliminary results on their recognition by yeast bearing specific membrane lectins.



DMPO **PBN**

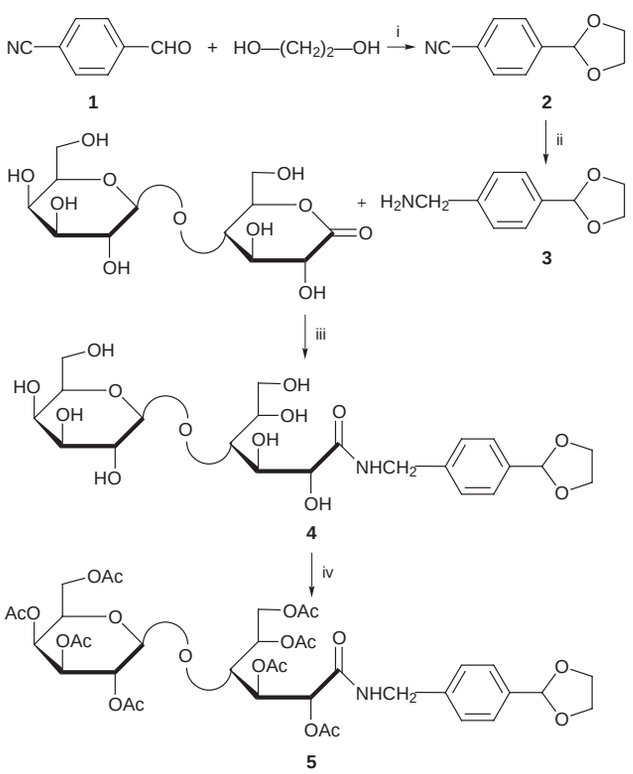


DEPMPO **PPN**

Results

Synthesis

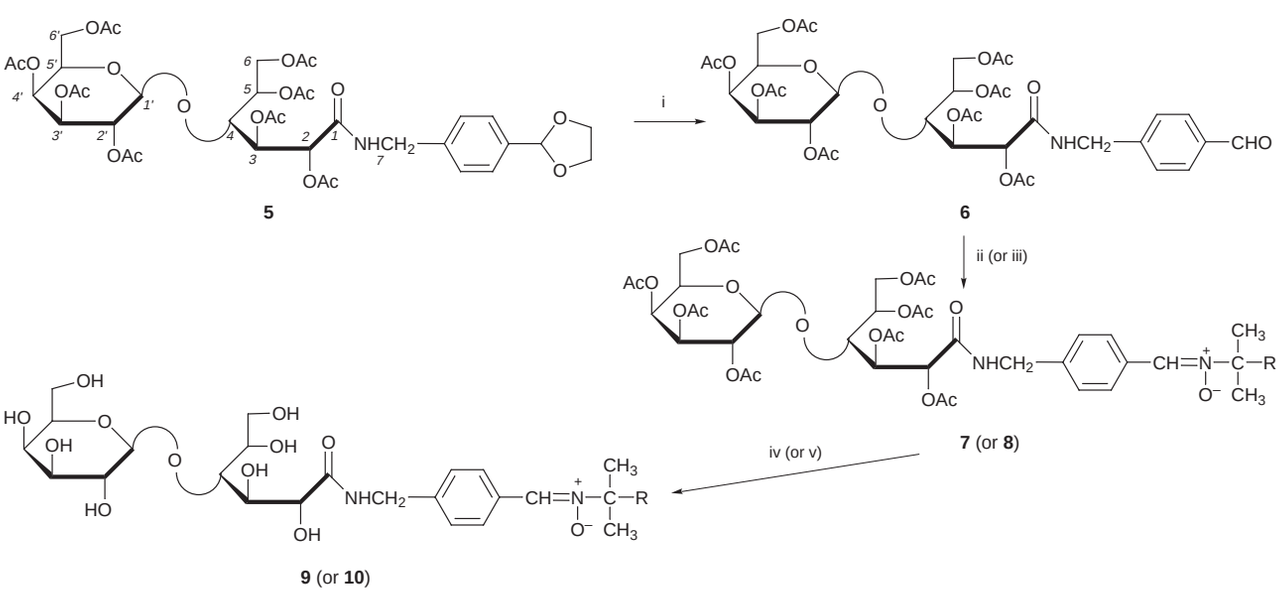
N-[4-(1,3-Dioxacyclopent-2-yl)benzyl]lactobionamide (**4**) was prepared from 4-cyanobenzaldehyde (**1**) and lactobiono-1,5-lactone[27] in a four-step sequence as indicated in Scheme 1. The condensation of this lactone with 4-(1,3-dioxacyclopent-2-yl)-benzylamine (**3**) was carried out at 50 °C in methanol using catalytic amounts of triethylamine (TEA) and the experimental conditions described by Williams *et al.*[28] In order to make easier the purification of compound **4**, its hydroxy functions were first acetylated. The peracetylated derivative **5** was purified by silica gel column chromatography. Then transacetalisation with acetaldehyde afforded the corresponding aldehyde **6** in good yield (Scheme 2). Nitrones **7** and **8** were obtained following a well known procedure[29] which consists of condensing *N*-*tert*-butylhydroxylamine or diethyl (1-hydroxyamino-1-methylethyl)phosphonate[30] respectively with **6**. The best yields were obtained carrying out the condensation in the dark, using THF as solvent under an inert atmosphere. Nitrones **7** and **8** were purified by column chromatography on silica gel and on LH 20 Sephadex resin. Hydrolysis of the acetyl group using catalytic amounts of sodium methoxide (for **7**) or sodium ethoxide (for **8**) led in quantitative yields to the highly water soluble nitrones LAMPBN (**9**) and LAMPPN (**10**).



Scheme 1 Synthesis of *N*-[4-(1,3-dioxacyclopent-2-yl)benzyl]octa-*O*-acetyllactobionamide **5**: i, TsOH, $PhCH_3$; ii, $AlLiH_4$, Et_2O; iii, TEA catalyst MeOH; iv, Ac_2O, pyridine.

Superoxide spin-trapping studies in phosphate buffer

With LAMPPN (10). When the superoxide radical, $O_2^{\bullet -}$, was generated in phosphate buffer at acidic pH (5.6) using the hypoxanthine–xanthine oxidase system in the presence of LAMPPN (**10**) (0.1 mol dm^{-3}) the ESR spectrum shown in Fig. 1(a) was observed during the first minutes of the experiment. The main signal is a 12 line signal attributable to a nitroxide ($a_P = 4.18$ mT, $a_N = 1.34$ mT, $a_H = 0.225$ mT, $g = 2.0060$) which was suppressed in the presence of superoxide dismutase [SOD, 90 unit cm^{-3}, Fig. 1(e)]. The spectrum of Fig. 1(a) was assigned to the LAMPPN–superoxide spin adduct, LAMPPN–OOH. It is worthy of note that the ESR parameters of LAMPPN–OOH are very close to those of the PPN–superoxide spin adduct.[20] With the inclusion of glutathione peroxidase (GPX, 10 unit cm^{-3}) and small amounts of reduced glutathione (GSH, 0.3 mmol dm^{-3}) in the reaction mixture the reduction of LAMPPN–OOH to LAMPPN–OH was expected.[31] However, when we added GPX and GSH to the reaction mixture the LAMPPN–OOH signal was replaced by a 50 min persistent broad signal [$a_P = 4.20$ mT, $a_N = 1.45$ mT, $a_H = 0.34$ mT, Fig. 1(f)] characteristic of the decomposition of the LAMPPN–OH spin adduct (results not shown). The ESR signal of the LAMPPN–OOH spin adduct lasted approximately 8 min at pH 5.6 and then, only a very broad persistent signal hereafter named **A** ($a_P = 4.16$ mT, $a_N = 1.40$ mT, a_H unresolved) was observed. The formation of **A** was not affected by the inclusion of SOD in the reaction mixture. At pH 7, a signal of the LAMPPN–OOH spin adduct was also detected but it was



Scheme 2 Synthesis of LAMPBN **9** or LAMPPN **10**: i, CH_3CHO, TsOH catalyst; ii, Bu^{t}NHOH, THF; iii, $(CH_3)_2[P(O)(OEt)_2]CNHOH$, THF; iv, MeONa, MeOH; v, EtONa, EtOH.

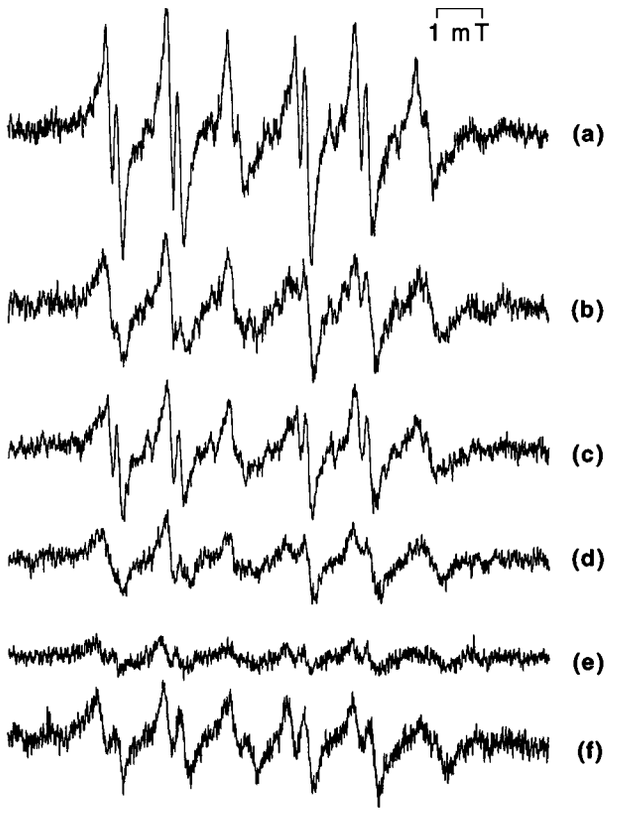


Fig. 1 ESR spectra observed after superoxide generation with XO–HX system in the presence of LAMPPN **10** and DTPA, in phosphate buffer (a) at pH 5.6, 1 min after mixing, (b) at pH 5.6, 17 min after mixing, (c) at pH 7, 1 min after mixing, (d) at pH 7, 10 min after mixing, (e) at pH 5.6, with addition of SOD, (f) at pH 5.6, with addition of GSH and GPX.

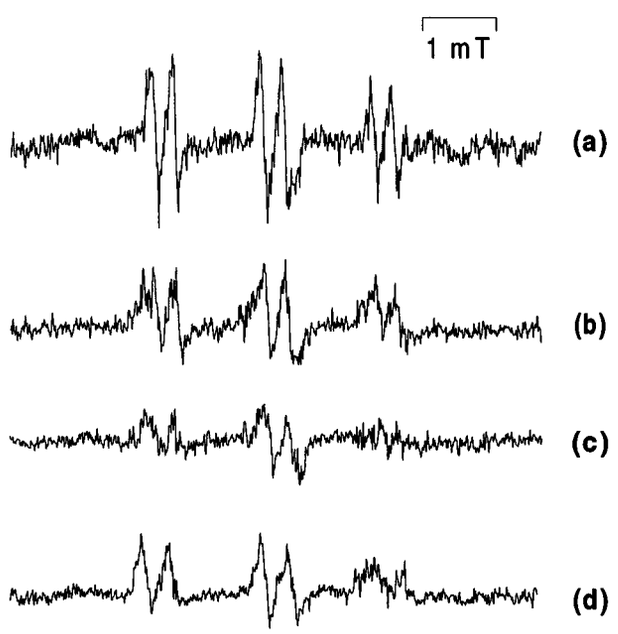


Fig. 2 ESR spectra observed after superoxide generation with XO–HX system in the presence of LAMPBN **9** and DTPA, in phosphate buffer at pH 5.6, (a) 1 min after mixing, (b) 3 min after mixing, (c) 5 min after mixing, (d) 1 min after mixing with addition of SOD.

weaker, and after 4 min only the signal **A** was observable. At pH 8.2, no significant signals were observed.

With LAMPBN (9). The same experiments were conducted in the presence of the nitrone LAMPBN (**9**) and yielded the ESR spectra shown in Fig. 2. At pH 5.6, a weak signal exhibiting hfsc ($a_N = 1.48$ mT, $a_H = 0.287$ mT and $g = 2.0057$) very

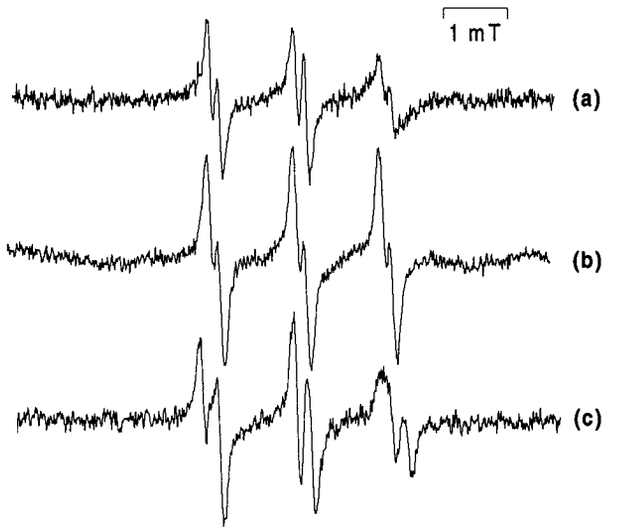


Fig. 3 ESR spectra observed (a) 9 min after mixing LAMPBN (0.1 mol dm^{-3}) and H_2O_2 (1%) in pyridine, (b) 6 min after mixing PBN (0.1 mol dm^{-3}) and H_2O_2 (1%) in pyridine, (c) 30 min after a ten-fold dilution of a pyridine solution of LAMPBN (1 mol dm^{-3}) and H_2O_2 (10%).

close to those of the PBN–superoxide spin adduct[32] was observed [Fig. 2(a) and (b)] and was assigned to the LAMPBN–OOH spin adduct. The signal disappeared swiftly and after 5 min, only a very poor signal **B** with higher a_N and a_H values ($a_N = 1.59$ mT, $a_H = 0.344$ mT) was detected [Fig. 2(c)]. In the presence of SOD, LAMPBN–OOH formation was stopped while the second signal **B** was bigger. On the other hand, the introduction of GPX (10 unit cm^{-3}) and GSH (0.3 mmol dm^{-3} for nitrone 0.1 mol dm^{-3}) led to superimposition of **B** and a new weak signal corresponding to the LAMPBN–OH spin adduct ($a_N = 1.55$ mT, $a_H = 0.27$ mT). At neutral pH, the signal **B** was largely predominant, whereas at basic pH no significant signal was observed.

It is worthy of note that we were not able to detect the superoxide adduct when the radical $O_2^{\bullet -}$ was generated by irradiating riboflavin in the presence of an electron donor (DTPA) in phosphate buffer containing either LAMPBN or LAMPPN.

For comparison the LAMPBN–OOH spin adduct was also generated by nucleophilic addition of hydroperoxide (1%) to LAMPBN (0.1 mol dm^{-3}) in pyridine, followed by oxidation of the ensuing hydroxylamine [Fig. 3(a)]. In pyridine, the adduct lasted more than twenty hours, and exhibited the following ESR parameters: $a_N = 1.34$ mT, $a_H = 0.169$ mT and $g = 2.0061$. Under similar experimental conditions, PBN led to an adduct [Fig. 3(b)] with almost similar hfsc ($a_N = 1.35$ mT, $a_H = 0.170$ mT and $g = 2.0061$). In order to measure the ESR parameters of LAMPBN–OOH in water we tried to prepare a higher concentration in pyridine with a view to transferring it to water. However, increasing the concentrations of LAMPBN up to 1 mol dm^{-3} and hydroperoxide up to 10%, afforded a new nitroxide **C** [$a_N = 1.467$ mT, $a_H = 0.218$ mT, Fig. 3(c)]. If **C** is transferred to water, significant changes of its hfsc values were observed ($a_N = 1.569$ mT and $a_H = 0.387$ mT).

Recognition by yeast lectins

In order to determine the lectin recognition ability of these new lactobionamide derivatives, we measured their inhibiting properties towards well-established yeast flocculation processes involving lectins. Yeast flocculation results from cell–cell recognition mechanisms involving interaction of an appropriate membrane lectin of a cell with carbohydrates, oligosaccharides or glycosidic structures localised on the surface of other cells.[33] When lectins are removed from yeast cell membranes,

Table 1 Inhibitory concentration of yeast *Kluyveromyces bulgaricus* flocculation (ICF) observed for different galactosyl derivatives.

Carbohydrate		ICF/mM
Monosaccharides	D-Galactose	3.12
	D-Mannose	No inhibition observed
	D-Glucose	No inhibition observed
Disaccharides	Lactose	1.25
	Melibiose	0.60
	Saccharose	No inhibition observed
Galactose derivatives	Methyl β-D-galactose	1.75
	PNP β-D-galactoside	0.83
	LAMPBN	**0.25**
	LAMPPN	**0.26**

flocculation of these cells in suspension is suppressed. If the extracted lectins are reintroduced in these suspensions, cell ability to aggregate can be restored unless the lectins were previously incubated with glycosidic agonists.[33] The agonist concentration which is needed to inhibit this reflocculation is a measure of the agonist affinity for the lectin. Aggregation of the yeast *Kluyveromyces bulgaricus* involves a galactose-specific lectin Kb CWL1. The flocculation degree can be reasonably estimated by direct or optical microscope observation. After growth, the flocculent yeast cells are harvested and the lectins are extracted using chelating agents or surfactants. As shown in Table 1, the affinities of products **9** and **10** measured as described above are much higher than those of free galactose and other galactosyl derivatives.

Discussion

Spin-trapping studies

The two nitrones **9** and **10** appeared poor scavengers of the superoxide radical. However, for **10** the presence of the β-phosphoryl group slightly increased the half-life of the corresponding superoxide spin adduct compared to that of the same adduct generated from **9**. The same trend was previously mentioned for the superoxide spin adducts obtained with PBN and PPN.[20] The LAMPBN–OOH and LAMPPN–OOH spin adducts exhibited coupling constants similar to those of PBN–OOH[32] and PPN–OOH[20] respectively. Furthermore, the direct decay of these spin adducts did not yield significant amounts of other paramagnetic species.

The ESR signals **A** [Fig. 1(b) or (d)] and **B** [Fig. 2(c) and (d)] were persistent and could be assigned to carbon-centred radical spin adducts, considering the coupling constant values of analogous spin adducts obtained with PPN and PBN.[21] For comparison, the radical $^{\bullet}CH_2OH$ was trapped with **9** and **10**, and the spectra of the resulting spin adducts are shown in Fig. 4. For the two nitrones the spin adducts yielded signals which were far more intense and much more persistent than those obtained by trapping oxygen-centred radicals. The coupling constants of LAMPBN–CH_2OH ($a_N = 1.59$ mT, $a_H = 0.373$ mT) and LAMPPN–CH_2OH ($a_P = 4.29$ mT, $a_N = 1.46$ mT, $a_H = 0.335$ mT) are very close to those observed for **B** and **A** respectively.

The formation of **A** and **B** was not observed in the absence of the superoxide generating system and we checked that **A** and **B** did not originate from the degradation of nitrones during their storage either in phosphate buffers or water. On the other hand, the formation of spin adducts resulting from the trapping of carbon-centred radicals was not detected when the superoxide was trapped either with PBN or PPN. The formation of **A** and **B** observed with **9** or **10** respectively could then result from the scavenging of radicals formed from the lactobionamide moiety. To support this assumption we trapped superoxide with PBN or PPN in the presence of equimolar amounts of *N*-isopropyllactobionamide. In both cases the decay of the superoxide spin adduct signal was accompanied by the growth of a new persistent signal corresponding to coupling constants ($a_N = 1.61$ mT and $a_H = 0.33$ mT for PBN and $a_P = 4.4$ mT, $a_N = 1.40$ mT, a_H unresolved for PPN) very close to those of **A** or **B**. The formation of free radicals from the glycosidic chain can not be related to the direct reactivity of superoxide and more likely arises from pseudo-Fenton reactions, as supported by the significant increase of **B** in the presence of SOD. Lactobionamides are able to chelate iron ions[34] and could compete in this respect with DTPA (diethylenetriaminepentaacetic acid), which is used to minimise $HO^{\bullet}$ formation in biological media containing $O_2^{\bullet -}$ and the derived H_2O_2. In our experiments a tiny production of $HO^{\bullet}$ close to the glycosidic chain could result in the formation of α-hydroxycarbon-centred radicals. As suggested by Isbell *et al.*, hydroxyl radicals involved in the degradation of alditols by hydrogen peroxide in basic media[35] could also be produced by a direct reaction of superoxide with H_2O_2.[36] The reaction of $HO^{\bullet}$ with carbohydrate leads to several carbon-centred radicals[37] and that could explain the large ESR line width observed for **A** and **B**.

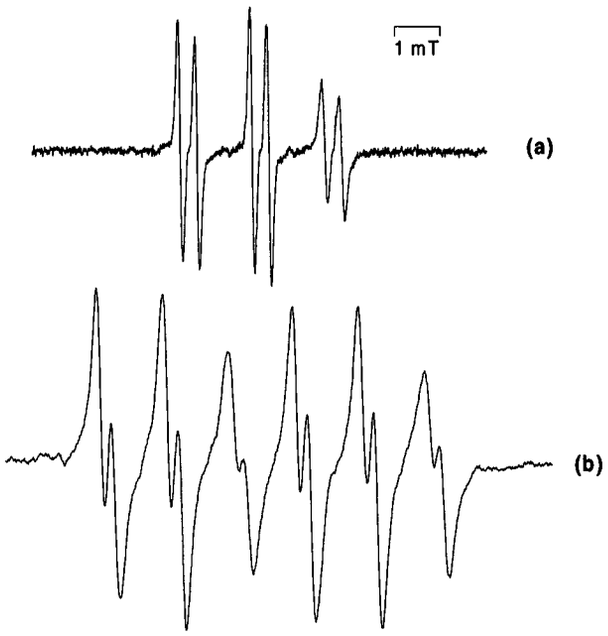
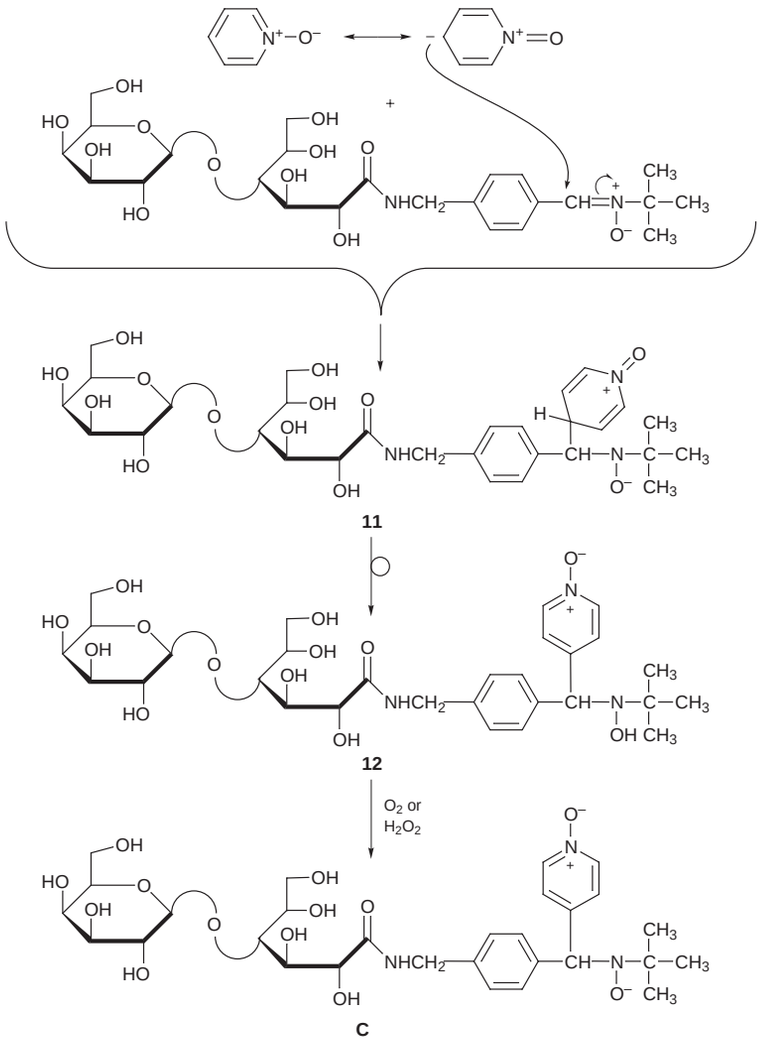


Fig. 4 ESR spectra observed after hydroxyl radical generation with H_2O_2–$FeSO_4$–EDTA system in phosphate buffer at pH 7 in the presence of 10% methanol and (a) LAMPBN **9**, (b) LAMPPN **10**.

As observed for many superoxide spin adducts obtained with nitrones,[38] the lifespan of LAMPBN–OOH was much longer in an organic solvent such as pyridine. The formation of radical **C** in the presence of a high concentration of hydrogen peroxide in pyridine could be tentatively explained as shown in Scheme 3. The oxidised pyridine could add to the nitrone to give the intermediate **11**, and then a proton shift yields the hydroxylamine **12** which is oxidised to the radical LAMPBN–[4-(*N*-oxy)pyridyl] **C**. To support the proposed mechanism, we can mention that the hfsc values of **C** are very close to those reported for the nitroxide PBN–[4-pyridyl] ($a_N = 1.444$ and $a_H = 0.217$ mT in pyridine and $a_N = 1.573$ and $a_H = 0.357$ in water)[39] and those that we measured for PBN–[4-(*N*-oxy)-pyridyl] ($a_N = 1.457$, $a_H = 0.222$ mT and $g = 2.0058$ in pyridine). These nitroxides were obtained indirectly by addition of a phenyl radical to α-(4-pyridyl) *tert*-butyl nitrone and α-(4-pyridyl 1-oxide) *tert*-butyl nitrone respectively. On the other hand Reszka *et al.*[38a] mentioned that a DMPO adduct of an unidentified carbon-centred radical was formed in pyridine in the presence of 30% hydrogen peroxide. This spin adduct does not appear when the reaction is carried out in benzene. The nucleophilic addition–oxidation mechanism (Forrester–Hepburn mechanism[40a]) has been established in benzene for PBN and a range of N-heteroatomic bases such as indoles



Scheme 3

(attack at position 3 of the indole moiety) in the presence of a weak oxidant.[40b]

Recognition by yeast lectins

The cell targeting ability of the galactoryl moiety of LAMPBN and LAMPPN is conserved and is even more pronounced if compared with free galactose. For the two nitrones the polyol chain acts as a rigid spacer arm which preserves the accessibility of the galactopyranose moiety to the specific lectin receptor. The more lipophilic part of the nitrones could also enhance their fusion to the lipophilic yeast cell membranes into where the lectins are anchored. Similar results have been observed with different galactosylated substrates bearing a hydrophobic moiety on their anomeric carbon.[41]

Conclusions

We have prepared two new nitrones bearing a lactobionamide moiety and we have shown that these nitrones are efficiently recognised by galactose-specific yeast lectins.

Our spin-trapping results suggest that linkage of a glycosyl group to the traps PBN and PPN does not modify significantly their ability to scavenge the superoxide radical in phosphate buffer. At physiological pH, the spin adducts are short lived. However, a diethoxyphosphoryl group on the α-carbon of the nitronyl function stabilises the superoxide spin adduct and the LAMPPN–OOH adduct was found to be significantly more persistent than the LAMPBN–OOH adduct. The formation and persistence of these adducts are strongly pH-dependent with a preference for acidic pH. We have shown that carbon-centred radicals are formed from the lactobionamide moiety and presumably result from the attack of $^{\bullet}OH$ radicals. However, the signals of the corresponding spin adducts do not hamper the identification of LAMPBN–OOH or LAMPPN–OOH. Compounds **9** and **10** are the first members of a new series of free radical scavengers able to target specific cells. In order to allow these new traps to cross the cell membranes, work is in progress to modulate the hydrophilic–lipophilic balance by inserting suitable hydrocarbon or perfluorocarbon chains into the molecules.

Experimental

Chemicals

THF and toluene were dried on sodium and benzophenone whereas methanol, ethanol and ethoxyethanol were dried on magnesium, and then solvents were distilled under an inert atmosphere. Diethyl (1-hydroxyamino-1-methylethyl)phosphonate was recrystallized and dried under high vacuum before each use. Melting points were taken on a Büchi capillary apparatus and have been left uncorrected. NMR spectra were obtained on Brucker AC 100 (^{31}P NMR 40.5 MHz), Brucker AC 200 (^{1}H NMR 200 MHz and ^{13}C NMR 50.3 MHz), and Brucker AM 400 X (^{1}H NMR 400 MHz and ^{13}C NMR 100.6 MHz) spectrometers. The interpretation of the spectra was achieved by comparison of heteronuclear ^{13}C–^{1}H chemical shift

correlation, and ^{1}H homonuclear correlation (*cf.* Scheme 2 for numbering). NMR coupling constant values are given in Hz and chemical shifts in ppm. $[\alpha]_D$ optical rotation values are given in 10^{-1} deg cm^2 g^{-1}. Mass spectra were recorded in the FAB$^+$ or LSIMS$^+$ mode on a ZabSpec TOF Micromass spectrometer.

4-(1,3-Dioxacyclopent-2-yl)benzonitrile 2. To a solution of 4-cyanobenzaldehyde (25 g, 0.190 mol) in 200 cm^3 of toluene, ethylene glycol (42.2 cm^3, 0.768 mol) and toluene-*p*-sulfonic acid (0.02 g, 0.10 mmol) were added. The mixture was refluxed under stirring during 9 h and the water was removed by azeotropic distillation by means of a Dean–Stark trap. The solution was cooled at 20 °C and 40 cm^3 of a 5% sodium bicarbonate aqueous solution were added. The organic layer was extracted and washed twice with 25 cm^3 of water. The product was dried on $MgSO_4$ and the solvent was evaporated under reduced pressure. Pure colourless hygroscopic 4-(1,3-dioxacyclopent-2-yl)benzonitrile **2** (29.75 g, 90%) was crystallized from the residual oil in pentane–diethyl ether (6:4). Mp 40 °C. Found: C, 68.60; H, 5.61; N, 7.95. $C_{10}H_9NO_2$ requires C, 68.56; H, 5.17; N, 7.99%. δ_H (200.13 MHz; $CDCl_3$; Me_4Si) 7.67 (2H, d, J_{AB} 8.2, 2 ArH_A), 7.58 (2H, d, J_{AB} 8.4, 2 ArH_B), 5.84 (1H, s), 4.2–4.0 [4H, AA′BB′, $(CH_2O)_2$]. δ_C (50.32 MHz; $CDCl_3$; Me_4Si) 194.1 (CN), 143.8 (1 C^{IV} Ar.), 137.5 (1 C^{IV} Ar.), 132.2 (2 C^{III} Ar.), 127.1 (2 C^{III} Ar.), 103.2 (1C, CH), 65.4 (2C, 2 CH_2).

4-(1,3-Dioxacyclopent-2-yl)benzylamine 3. To a well-stirred solution of $LiAlH_4$ (12.15 g, 0.32 mol) in 50 cm^3 of dry Et_2O was added dropwise at 0 °C under an inert atmosphere, 4-(1,3-dioxacyclopent-2-yl)benzonitrile **2** (28 g, 0.16 mol) in 100 cm^3 of Et_2O. The mixture was stirred at 0 °C during 4 h, then 12 h at room temperature. The mixture was hydrolysed by 95% ethanol (30 cm^3) and then by 20 cm^3 of ethanol–water (1:1). The ethereal supernatant layer was separated and the aqueous layer was evaporated to dryness. The resulting solid was extracted with 75 cm^3 of CH_2Cl_2. The combined organic layers were washed with 40 cm^3 of water and dried on Na_2SO_4. 4-(1,3-Dioxacyclopent-2-yl)benzylamine **3**, a yellow oil, was obtained (17.7 g, 62%) after evaporation of the solvent. δ_H (200.13 MHz; $CDCl_3$; Me_4Si) 7.41 (2H, d, J_{AB} 8.09, ArH_A), 7.27 (2H, d, J_{AB} 8.05, ArH_B), 5.75 (1H, s, CH), 4.2–3.9 (4H, AA′BB′, 2 OCH_2), 3.94 (2H, s, CH_2N), 2.18 (2H, s, NH_2). δ_C (50.32 MHz; $CDCl_3$; Me_4Si) 144.0 (1C^{IV} Ar.), 137.8 (1 C^{IV} Ar.), 127.0 (2 C^{III} Ar.), 126.6 (2 C^{III} Ar.), 103.5 (1C, CH), 65.2 (2 C, CH_2O), 46.0 (1C, CH_2N). Because of the high hygroscopy of **3**, the elementary analysis of the corresponding amine picrate is given here. The picrate was obtained after recrystallisation of the solid formed by adding picric acid to a saturated ethanolic solution of **3**. Found: C, 47.01; H, 3.85; N, 13.72. $C_{16}H_{16}N_4O_{10}$ requires C, 47.06; H, 3.94; N, 13.72%.

***N*-[4-(1,3-Dioxacyclopent-2-yl)benzyl]octa-*O*-acetyllactobionamide 5.** Lactobionic acid (17 g, 47.44 mmol) dissolved in anhydrous ethoxyethanol (60 cm^3) and toluene (5 cm^3) was stirred and evaporated at 45 °C under a reduced and dry atmosphere. The operation was repeated three times and the lactone (a white powder) was used quickly. Under argon, lactobionolactone (16.1 g, 47.31 mmol) was dissolved in 50 cm^3 of dry MeOH and was added slowly to a mixture of 4-(1,3-dioxacyclopent-2-yl)benzylamine **3** (6.8 g, 38 mmol) and triethylamine (0.5 cm^3, 3.6 mmol). The reaction mixture was refluxed during 3 h. The solvent was removed and the resulting crude *N*-[4-(1,3-dioxacyclopent-2-yl)benzyllactobionamide **4** (brown powder; 16.6 g, 85%) was acetylated without purification, by dropwise addition of acetic anhydride in pyridine (250 cm^3, 1:1) at 0 °C. The mixture was further stirred at room temperature during 18 h. The solution was poured in 500 cm^3 of cold water, and extracted three times with 50 cm^3 of CH_2Cl_2. The organic layer was washed with 1 M HCl then with water, and was dried on $MgSO_4$. This layer was evaporated to afford a solid foam. Purification of this product by silica gel column chromatography, eluting with a gradient of ethyl acetate (70% max.) in diethyl ether, lead to pure *N*-[4-(1,3-dioxacyclopent-2-yl)benzyl]octa-*O*-acetyllactobionamide **5** as a white powder (15.2 g, 53% yield with regard to **3**). Decomposition temperature (T_{dec}) 75 °C. Found: C, 53.41; H, 5.77; N, 1.63. $C_{38}H_{49}NO_2$ requires C, 53.33; H, 5.77; N, 1.60%. δ_H (400.13 MHz; $CDCl_3$; Me_4Si) 7.35 (2H, d, J_{AB} 8.0, ArH_A), 7.18 (2H, d, J_{AB} 8.0, ArH_B), 6.52 (1H, t, $J_{NH,7}$ 5.8, NH), 5.69 (1H, s, CH_2), 5.57 (1H, d, $J_{2,3}$ 6.1, H-2), 5.51 (1H, dd, $J_{3,2}$ 6.0, $J_{3,4}$ 3.7, H-3), 5.27 (1H, dd, $J_{4',3'}$ 3.3, H-4′), 5.08 (1H, dd, $J_{2',3'}$ 10.3, $J_{2',1'}$ 8.1, H-2′), 4.95–5.01 (1H, m, H-5), 4.90 (1H, dd, $J_{3',2'}$ 10.4, $J_{3',4'}$ 3.5, H-3′), 4.55 (1H, d, $J_{1',2'}$ 7.9, H-1′), 4.49–4.40 (1H, m, H-6_A + H-7_A), 4.28 (1H, dd, $J_{7,NH}$ 5.4, J_{AB} 14.9, H-7_B), 4.24 (1H, dd, $J_{3,4}$ 3.7, $J_{4,5}$ 6.5, H-3), 4.09–3.90 (7H, m, 2 CH_2O + H-6_B + H-6′$_A$ + H-6′$_B$), 3.78 (1H, t, $J_{5',6'}$ 6.6, H-5′), 2.06, 2.00, 1.99, 1.96, 1.95, 1.91 and 1.89 (24H, 7s, CH_3CO_2-). δ_C (100.61 MHz; $CDCl_3$; Me_4Si) 170.40, 170.10, 169.96, 169.67, 169.54 and 169.20 (8C, 8 -CO_2CH_3), 167.0 (C-1), 138.0 (1C^{IV} Ar.), 137.0 (1C^{IV} Ar.), 127.0 (1C^{III} Ar.), 126.0 (1C^{III} Ar.), 103.0 (CH), 101.0 (C-1′), 77.2 (C-4),71.6 (C-2), 70.9 (C-3′ and C-5′), 69.7 (C-5), 69.1 (C-2′), 68.9 (C-3), 66.7 (C-4′), 65.2 (2C, CH_2), 61.5 (C-6), 60.9 (C-6′), 42.9 (C-7), 20.76, 20.69, 20.62, 20.55, 20.53 and 20.42 (8C, CH_3CO_2-).

***N*-(4-Formylbenzyl)octa-*O*-acetyllactobionamide 6.** The amide **5** (7 g, 8.18 mmol) and toluene-*p*-sulfonic acid (20 mg, 0.15 mmol) were dissolved in acetaldehyde (50 cm^3, 1.07 mol). The reaction mixture was stirred at 15 °C during 7 h, then evaporated at 35 °C several times with at each time the addition of 30 cm^3 of CH_2Cl_2 to eliminate trimethyltrioxane. The resulting product was dissolved in CH_2Cl_2 and washed with 15 cm^3 of 5% $NaHCO_3$ aqueous solution then with 15 cm^3 of water, and it was further dried on Na_2SO_4. The solvent was removed under reduced pressure and the solid was purified by washing in pentane–diethyl ether (4:1) under sonication, and recovered by filtration. *N*-(4-Formylbenzyl)octa-*O*-acetyllactobionamide **6**, a white powder, was again washed in a Soxhlet system with pentane (5.17 g, 86%). T_{dec} 75 °C. δ_H (200.13 MHz; $CDCl_3$; Me_4Si) 9.99 (1H, s, -CHO), 7.85 (2H, d, J_{AB} 8.2, ArH_A), 7.41 (2H, d, J_{AB} 8.2, ArH_B), 6.67 (1H, t, $J_{NH,7}$ 5.8, -NH), 5.75–3.90 (15H, m), 2.17, 2.16, 2.08, 2.05, 2.04, 2.02, 1.98 (24H, 7s, CH_3CO_2-). δ_C (50.32 MHz; $CDCl_3$; Me_4Si) 191.7 (-CHO), 170.5, 169.8, 169.7, 169.3 and 167.4 (8 -$COCH_3$), 167.4 (-CONH), 145.1 (1C^{IV} Ar.), 137.0 (1C^{IV} Ar.), 130.1 (2C^{III} Ar.), 127.9 (2C^{III} Ar.), 101.6 (C-1′), 77.3 (C-4), 71.0 (C-2), 70.8 (C-5′ and C-3′), 69.25 (C-5), 69.0 (C-3), 68.9 (C-2′), 66.7 (C-4′), 61.5 (C-6), 60.9 (C-6′), 42.9 (C-7), 20.8, 20.7, 20.64, 20.55 and 20.44 (8 CH_3CO_2-). High resolution mass spectra found, 812.263; $C_{36}H_{45}NO_{20}$ requires 812.2613; *m/z* (LSIMS Cs^+, 8 kV) 812.8 (22%, $[M + H]^+$), 464.5 (5), 331.3 (100), 289.3 (18), and 154.2 (82).

***N*-[4-(Octa-*O*-acetyllactobionamidomethylene)benzylidene]-*N*-*tert*-butylamine *N*-oxide 7.** *N*-*Tert*-butylhydroxylamine was extracted with Et_2O after the addition of a saturated $NaHCO_3$ aqueous solution to *N*-*tert*-butylhydroxylamine hydrochloride (0.58 g, 4.61 mmol) until pH 8. The organic layer was dried with Na_2SO_4 and the solvent was evaporated. Under argon *N*-*tert*-butylhydroxylamine (0.334 g, 3.75 mmol) dissolved in 5 cm^3 of anhydrous THF was added to compound **6** (3 g, 3.70 mmol) in 15 cm^3 of anhydrous THF. The mixture was stirred in the dark at 50 °C during 120 h with the addition of *N*-*tert*-butylhydroxylamine (0.1 g, 1.12 mmol) and degassed 3 Å molecular sieves (0.2 g) every 48 h. The emerald green colour of the reaction mixture traduced the presence of nitroso compounds, resulting from the auto-oxidation of the hydroxylamine. The mixture was filtrated and the solvent was removed until the formation of a solid foam which was triturated in diethyl ether to give, after filtration, a white powder. This solid was purified by flash silica gel column chromatography, eluting with Et_2O with a gradient of ethyl acetate and acetonitrile (50 and 10% max. respectively). The nitrone **7** was obtained as a white hygroscopic powder (1.99 g, 58%). T_{dec} 65 °C. δ_H (200.13 MHz; $CDCl_3$; Me_4Si) 8.29 (2H, d, J_{AB} 8.4, ArH_A), 7.57 (1H, s,

-CH=NO-), 7.32 (2H, d, J_{AB} 8.5, ArH_B), 6.56 (1H, t, J 5.8, –NH), 5.72-3.75 (15H), 2.23, 2.19, 2.18, 2.12, 2.08, 2.07, 2.03 and 2.01 (24H, 8 s, CH_3CO_2), 1.64 (9H, 1 s, tBu). δ_C (50.32 MHz; $CDCl_3$; Me_4Si) 170.5, 170.4, 169.9, 169.7, 169.6 and 169.4 (8C, -CO_2CH_3), 167.4 (-CONH-), 140.1 ($1C^{IV}$ Ar.), 135.0 (CH=NO-), 130.1 ($1C^{IV}$ Ar.), 129.1 ($2C^{III}$ Ar.), 127.6 ($2C^{III}$ Ar.), 102.0 (C-1′), 77.3 (C-4), 71.0 (C-2), 70.8 (C-5′ and C-3′), 69.2 (C-5), 69.0 (C-3), 68.9 (C-2′), 66.7 (C-4′), 61.5 (C-6), 60.9 (C-6′), 42.9 (C-7), 53.4 [$C(CH_3)_3$], 28.2 [3C, $(CH_3)_3C$], 20.8, 20.7, 20.6 and 20.4 (8C, CH_3CO-). High resolution mass spectra found, 883.3330; $C_{40}H_{55}N_2O_{20}$ requires 883.3348; *m*/*z* (LSIMS Cs^+, 8 kV), 883.0 (100%, $[M + H]^+$), 867.3 (17, $[(M + H) - O]^+$), 553.5 (28), 329.5 (82).

***N*-[4-(Lactobionamidomethylene)benzylidene]-*N*-*tert*-butylamine *N*-oxide (LAMPBN) 9.** The nitrone **7** (1.8 g, 2.04 mmol) was stirred 1 h at 20 °C with sodium methoxide (0.005 g, 0.1 mmol) dissolved in 40 cm^3 of dry MeOH. The nitrone **9** was obtained as a white powder (1.08 g, 98%) after solvent evaporation and azeotropic distillation with acetonitrile of residual methanol. Paramagnetic ppm impurities became diamagnetic by storage of the nitrone in chelexed water and then lyophilisation. $[\alpha]_{20}^{D} = +16.8°$ (*c* 1 in CH_3OH). T_{dec} 65 °C. δ_H (400.13 MHz; CD_3OD; Me_4Si) 8.27 (2H, d, J_{AB} 8.4, 2ArHA), 7.88 (1H, s, CH=NO-), 7.42 (2H, d, J_{AB} 8.4, 2ArHB), 4.54 (1H, d, J_{AB} 15.6, H-7_A), 4.47 (1H, d, $J_{1',2'}$ 7.5, H-1′), 4.45 (1H, d, J_{AB} 15.6, H-7_B), 4.42 (1H, d, $J_{2,3}$ 2.3, H-2), 4.26 (1H, dd, $J_{3,2}$ 2.3, $J_{3,4}$ 4, H-3), 3.94 (1H, dd, $J_{4,3}$ 4.1, $J_{4,5}$ 6.5, H-4), 3.91 (1H, dt, $J_{5,4}$ 6.5, $J_{5,6}$ 3.3, H-5), 3.81–3.77 (3H, m, H-6_A + H-6_A + H-4′), 3.77 (1H, J_{AB} 11.5, $J_{6',5'}$ 7.5, H-6′$_A$), 3.67 (1H, J_{AB} 11.5, $J_{6',5'}$ 4.4 , H-6′$_B$), 3.6–3.51 (2H, m, H-5′ + H-2′), 3.47 (1H, dd, $J_{3',4'}$ 9.8, $J_{3',2'}$ 3.2, H-3′), 1.58 (9H, 1 s, 3 CH_3). δ_C (100.61 MHz; CD_3OD; Me_4Si) 175.36 (C-1), 143.35 ($1C^{IV}$ Ar.), 134.76 (CH=NO-), 130.99 ($2C^{III}$ Ar.), 130.71 ($1C^{IV}$ Ar.), 128.33 ($2C^{III}$ Ar.), 105.78 (C-1′), 83.36 (C-4), 77.22 (C-5′), 74.82 (C-3′ or C-2′), 74.06 (C-2), 73.22 (C-5), 72.83 (C-2′ or C-3′), 72.57 (C-3), 72.02 (C^{IV} of tBu), 70.38 (C-4′), 63.83 (C-6), 62.68 (C-6′), 43.49 (C-7), 28.38 [3C, $(CH_3)_3C$]. High resolution mass spectra found, 547.2518; $C_{24}H_{38}N_2O_{12}$ requires 547.2503; *m*/*z* (FAB^+, 8 kV), 547.4 (30%, $[M + H]^+$), 460.3 (2), 385.3 (30, $C_{18}H_{29}N_2O_7$, type Y fragment), 307.2 (18), 207.2 (4), 154.2 (100).

Diethyl [1-methyl-1-[4-(octa-*O*-acetyllactobionamidomethylene)benzylidene]azinoylethylphosphonate 8. Under an argon atmosphere, diethyl (1-hydroxyamino-1-methylethyl)phosphonate (0.90 g, 4.26 mmol) in 15 cm^3 of dry THF was added to *N*-(4-formylbenzyl)octa-*O*-acetyllactobionamide **6** (5.75 g, 7.08 mmol). Degassed molecular sieves 3 Å (0.4 g) and four equal portions of diethyl (1-hydroxyamino-1-methylethyl)phosphonate (2.15 g, 10.14 mmol in total) in 5 cm^3 of THF were added to the solution every 19 h under efficient stirring. The reaction was completed in 76 h at 40 °C. The green coloured reaction mixture was evaporated until the formation of a dry foam. The crude product was purified by silica gel column flash chromatography with *tert*-butyl methyl ether and a gradient of ethyl acetate and ethanol (50 and 20% max. respectively) as eluent. The product was also purified on a Sephadex LH-20 column with an ethanol–dichloromethane (1:1) eluent. The nitrone **8** was obtained as a white powder (4.2 g, 60%). T_{dec} 70 °C. δ_P (40.5 MHz; $CDCl_3$; H_3PO_4) 22.15. δ_H (200.13 MHz; $CDCl_3$; Me_4Si) 8.24 (2H, d, J_{AB} 8.28, 2ArHA), 7.74 (1H, d, $J_{H,P}$ 2.65, CH=NO-), 7.28 (2H, d, J_{AB} 8.28, 2ArHB), 6.52 (1H, t, $J_{H,H}$ 5.76, CONH), 5.72–3.62 (19H, m), 2.16, 2.15, 2.08, 2.07, 2.04, 2.01 and 1.98 (24H, 7s, 8 CH_3CO_2-), 1.83 [6H, d, J_{HP} 14.85, -$C(CH_3)_2$], 1.32 [6H, t, J_{HH} 7.07, -$(OCH_2CH_3)_2$]. δ_C (100.61 MHz; $CDCl_3$; Me_4Si) 170.49, 170.18, 170.04, 169.79, 169.75, 169.63 and 169.30 (8C, 7s, 8 -$COCH_3$), 167.25 (1C, s, -CONH-), 139.99 (1C, s, $1C^{IV}$ Ar.), 132.70 (1C, d, $J_{C,P}$ 5.03, CH=NO-), 130.16 ($1C^{IV}$ Ar.), 129.37 ($2C^{III}$ Ar.), 127.61 ($2C^{III}$ Ar.), 101.79 (C-1′), 77.25 (1C), 72.94 (1C, d, $J_{C,P}$ 153.93, -*C*-P), 71.70 (1C), 71.09 (1C), 71.03 (1C), 69.89 (1C), 69.29 (1C), 69.09 (1C), 66.88 (1C), 63.41 [2C, d, $J_{C,P}$ 6.74, -$(OCH_2CH_3)_2$], 61.72 (1C), 60.97 (1C), 43.10 (1C), 23.29 [2C, -$C(CH_3)_2$], 20.85, 20.78, 20.70, 20.62, 20.55 and 20.50 (8C, 8 CH_3CO-), 16.44 [2C, d, $J_{C,P}$ 5.93, -$(OCH_2CH_3)_2$]. High resolution mass spectra found, 1005.348; $C_{43}H_{62}N_2O_{23}P$ requires 1005.3481; *m*/*z* (LSIMS Cs^+, 8 kV) 1005.4 (100%, $[M + H]^+$), 989.4 (13, $[(M + H) - O]^+$), 675.3 (21), 331.0 (26).

Diethyl [1-methyl-1-[4-(lactobionamidomethylene)benzylidene]azinoylethyl]phosphonate (LAMPPN) 10. Under argon, the nitrone **8** (0.250 g, 0.25 mmol) in 20 cm^3 of dry ethanol was stirred 20 min at 20 °C in the presence of EtONa (0.1 mmol). Then two portions of 20 cm^3 of 95% ethanol were added. Solvent removal was completed by azeotropic distillation using acetonitrile and by drying under high vacuum. The nitrone LAMPPN **10** was obtained as a white powder (0.160 g, 98%). T_{dec} 80 °C. $[\alpha]_{20}^{D} = +13.3°$ (*c* 1 in CH_3OH). δ_P (40.5 MHz; CD_3OD; H_3PO_4) 26.38. δ_H (400.13 MHz; CD_3OD; Me_4Si) 8.21 (2H, d, J_{AB} 8.4, 2ArHA), 7.91 (1H, d, $J_{H,P}$ 2.4, CH=NO-), 7.43 (2H, J_{AB} 8.4, 2ArHB), 4.55 (1H, d, J_{AB} 15.7, H-7A), 4.47 (1H, d, J_{AB} 15.7, H-7B), 4.47 (1H, d, $J_{1',2'}$ 7.7, H-1′), 4.43 (1H, d, $J_{2,3}$ 2.2, H-2), 4.27 (1H, dd, $J_{3,2}$ 2.3, $J_{3,4}$ 4.0, H-3), 4.22 (2H, qd, $J_{H,H}$ 7.1, $J_{H,P}$ 3.5, -OCH_2CH_3), 4.20 (2H, qd, $J_{H,H}$ 7.1, $J_{H,P}$ 2.7, -OCH_2CH_3), 3.95 (1H, dd, $J_{4,5}$ 6.3, $J_{4,3}$ 4.2, H-4), 3.89 (1H, dt, $J_{5,4}$ 6.4, $J_{5,6}$ 4.2, H-5), 3.82–3.79 (3H, m, H-6A + H-6B + H-4′), 3.78 (1H, dd, J_{AB} 11.5, $J_{6',5'}$ 7.5, H-6′A), 3.68 (1H, dd, $J_{A,B}$ 11.5, $J_{6',5'}$ 4.4, H-6′B), 3.58–3.54 (2H, m, H-2′+ H-5′), 3.48 (dd, $J_{3',2'}$ 3.3, $J_{3',4'}$ 9.8, H-3′), 1.83 [6H, d, J_{HP} 15.3, -$C(CH_3)_2$], 1.33 [6H, t, J_{HH} 7.0, -$(OCH_2CH_3)_2$]. δ_C (100.61 MHz; CD_3OD; Me_4Si) 175.74 (C-1), 143.81 ($1C^{IV}$ Ar.), 135.92 (1C, d, $J_{C,P}$ 6.9, CH=NO-), 131.11 ($2C^{III}$ Ar), 130.80 ($1C^{IV}$ Ar), 128.58 ($2C^{III}$ Ar), 107.03 (C-1′), 83.63 (C-4), 77.4 (C-5′), 75.04 (C-3′), 74.91 [1C, d, $J_{C,P}$ 159.9, -$C(CH_3)_2$], 74.28 (C-2), 73.46 (C-5), 73.04 (C-2′), 72.88 (C-3), 70.62 (C-4), 65.14 [2C, d, $J_{C,P}$ 7.0, -$(OCH_2CH_3)_2$], 64.07 (C-6), 62.92 (C-6′), 43.71 (C-7), 23.76 [2C, -$C(CH_3)_2$], 16.95 [2C, d, $J_{C,P}$ 6.1, -$(OCH_2CH_3)_2$]. High resolution mass spectra found, 669.263; $C_{27}H_{46}N_2O_{15}P$ requires 669.2636; *m*/*z* (LSIMS Cs^+, 8 kV) 691.2 (100%, $[(M + Na - H) + H]^+$), 669.2 (36, $[M + H]^+$), 507.2 (49), 351.1 (8), 307.1 (14), 154.1 (82).

Spin-trapping studies

Xanthine oxidase (from cow's milk, phosphate-free) and superoxide dismutase (from bovine erythrocytes) were obtained from Bœringer Mannheim Biochemica Co. All chemicals and glutathione peroxidase (from bovine erythrocytes) were purchased from Sigma Chemical Co. To remove trace metal impurities, distilled water for aqueous solutions or buffers was stirred before use for 6 h in the presence of sodium iminodiacetate chelating resin (40 g dm^{-3}). ESR spectra were recorded on a Brucker ESP 300 spectrometer equipped with an NMR gaussmeter for magnetic field calibration. An HP 5350B microwave frequency counter was used for the determination of Landé factors *g*.

Spin-trapping of superoxide generated by the XO–HX system. The nitrone (0.1 mol dm^{-3}) was added to the $O_2^{\bullet -}$ generating system, composed of hypoxanthine (0.4 mmol dm^{-3}), DTPA (1 mmol dm^{-3}) and xanthine oxidase (0.4 unit cm^{-3}) in phosphate buffer (0.1 mol dm^{-3}) at different pH (5.6, 7 and 8.2). For each series of experiments the first spectrum was recorded 1 min after mixing the different components, the enzyme being the last product introduced into the solution. Inhibition of superoxide adduct formation was achieved by the addition of 90 unit cm^{-3} of superoxide dismutase. The superoxide spin adduct was reduced to the corresponding hydroxyl spin adduct with the use of GPX (10 unit cm^{-3}) in the presence of GSH (0.3 mmol dm^{-3}).

Nucleophilic addition of H_2O_2 in pyridine. For each series of experiments the first ESR spectrum was recorded 1 min after addition of H_2O_2 (1%) to a pyridine solution of nitrone (0.1 mol dm^{-3}) in pyridine or after a 10-fold dilution of a pyridine solution of nitrone (1 mol dm^{-3}) and H_2O_2 (10%).

Spin-trapping of $HOCH_2^{\bullet}$ radicals generated by the action of hydroxyl radical on methanol. $HO^{\bullet}$ radicals were generated in phosphate buffer (0.1 mol dm^{-3}) by a Fenton reaction (2 mmol dm^{-3} H_2O_2, 1 mmol dm^{-3} EDTA, ethylenediaminetetraacetic acid, and 2 mmol dm^{-3} $FeSO_4$) in the presence of methanol (10%) and the nitrone (0.1 mol dm^{-3}). For each series of experiments the first spectrum was recorded 1 min after mixing the different components.

Spin-trapping of phenyl radical, generated by UV-photolysis of iodobenzene, with α-(4-pyridyl 1-oxide) *tert*-butyl nitrone. The spectra were recorded after 30 s photolysis using a 600 W xenomercury arc (every wavelength) of a pyridine solution of iodobenzene (10%) and nitrone (0.1 mol dm^{-3}) in a quartz cell.

Interaction with lectin studies

Extraction of galactose-specific lectin from yeast cells. The Kb CWL1 lectin involved in the flocculation phenomenon of the yeast *Kluyveromyces bulgaricus*[42] was isolated from whole yeast cells. After growth in a 2 dm^3 fermentor under aerobic conditions, *K. bulgaricus* cells were harvested by centrifugation at 3000 *g* for 10 min at 4 °C, washed with Helm's buffer (150 mmol dm^{-3} CH_3COONa, 3.75 mmol dm^{-3} $CaCl_2$, 3 mM NaN_3) at pH 4.5, treated with a 0.2 mol dm^{-3} D-galactose solution and extensively washed with a 0.01 mol dm^{-3} phosphate buffer at pH 7. Then cells were suspended at a concentration of 4% (w/v) in PBS (phosphate buffered saline) supplemented with EDTA and were incubated at 37 °C for 90 min with light stirring. After centrifugation at 3000 *g* for 10 min, the supernatant was collected and dialysed at 4 °C for at least 48 h against distilled water, then freeze-dried. The residual flocculating activity of the yeast cells was assessed in Helm's acetate buffer.

Flocculation and flocculation inhibition tests. Non-flocculating yeast cells obtained after lectin extraction were washed first with distilled water, then with 1% EDTA aqueous solution and twice with distilled water. Washed cells (about 2.5×10^6 cells cm^{-3}) were suspended in 10 cm^3 Helm's acetate buffer. The lectin titre determination was assayed in glass tubes by mixing together 50 mm^3 of two-fold serial dilutions of lectin solution in Helm's buffer at pH 4.5 and 50 mm^3 of yeast Helm's buffer suspension. A positive reaction resulted in formation of aggregate (flocs) and the lectin titre corresponded to the highest dilution of the lectin dilution giving detectable aggregation of deflocculated yeasts. Reflocculation of the yeast cells was achieved in glass tubes by mixing 50 mm^3 of a lectin solution titre 4 with 50 mm^3 of a suspension of deflocculated yeasts. Inhibition of flocculation was assayed in glass tubes by mixing 50 mm^3 of two-fold serial dilution of LAMPBN or LAMPPN solutions in Helm's acetate buffer at pH 4.5 with 50 mm^3 of lectin solution titre 4. After an incubation of 60 min at room temperature, 50 mm^3 of deflocculated yeast suspension were added. Yeast flocculation was estimated by visual reading or optical microscope observation after 1 h incubation at room temperature. The inhibitory concentration of flocculation (ICF) corresponds to the tube which shows a visible aggregation phenomenon. Control of inhibition of flocculation was checked in the same way but using free galactose, methylgalactose and *p*-nitrophenylgalactose derivatives.

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